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Award Number: DAMD17-02-1-0611

TITLE: Mechanism of FADD-DN-Induced Apoptosis in Normal Breast Cells

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REPORT DATE: April 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20050819112

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE April 2005	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Apr 2002 - 31 Mar 2005)	
4. TITLE AND SUBTITLE Mechanism of FADD-DN-Induced Apoptosis in Normal Breast Cells			5. FUNDING NUMBERS DAMD17-02-1-0611	
6. AUTHOR(S) Lance R. Thomas, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Wake Forest University School of Medicine Winston-Salem, North Carolina 27157 <i>E-Mail:</i> Lance.thomas@vanderbilt.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) <p>Normal cells undergo apoptosis in response to inappropriate growth signals or the lack of overt survival signals. Tumor cells possess defects in apoptosis regulatory pathways and do not undergo apoptosis in these situations. Because FADD is an essential component of receptor mediated apoptosis, a dominant-negative version (FADD-DN) is able to block apoptosis induced by death ligands in many cell lines. While studying FADD signaling, our laboratory made the surprising discovery that FADD-DN can <u>induce</u> apoptosis in normal breast epithelial cells. Because FADD-DN induces apoptosis in normal but not cancerous breast epithelial cells, we hypothesize that FADD-DN interacts with one or more proteins expressed in breast epithelia. Since breast tumor cells do not die in response to FADD-DN, the potential FADD-DN interacting partners are likely to be involved in carcinogenesis. Since defects in apoptotic pathways are a prerequisite to cancer, understanding the nature of these defects may bring about potential treatments. FADD-DN signaling presents a novel apoptotic pathway that is fundamental in normal breast epithelia, but not breast cancer cells. Components of this pathway may identify potential therapeutic targets that allow the reactivation of this apoptotic response in cancer cells</p>				
14. SUBJECT TERMS FADD, apoptosis, TRAIL, DR5, reverse two-hybrid				15. NUMBER OF PAGES 7
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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Introduction

Normal cells undergo apoptosis in response to inappropriate growth signals or the lack of overt survival signals. Tumor cells possess defects in apoptosis regulatory pathways and do not undergo apoptosis in these situations. There are two modes of apoptosis - an intrinsic pathway initiated by stress such as DNA damage and an extrinsic pathway resulting from activation of death receptors. Binding of ligand to a death receptor such as Fas, TNFR1 or TRAIL receptors 1 and 2 leads to activation of that receptor. This results in the recruitment of the cytoplasmic adaptor protein FADD to the receptor complex and activation of caspase-8. Because FADD is an essential component of receptor mediated apoptosis, a dominant-negative version (FADD-DN) is able to block both Fas and TNF induced apoptosis in many cell lines. However, experiments in our lab indicate that FADD-DN can kill normal human breast epithelial cells but not breast tumor cells. Since the only known role of FADD is an adaptor molecule, our hypothesis is that FADD-DN induces apoptosis in normal breast cells through interactions with one or more proteins expressed in breast epithelia. Our approach is to identify proteins that bind to FADD then identify the subset that are involved in FADD-DN binding using mutational analysis. Because breast tumor cells do not die in response to FADD-DN, the potential FADD-DN interacting partners are likely to be involved in carcinogenesis.

Body

We have achieved all the objectives described in the approved statement of work for years one and two. From 17 FADD-DN interacting proteins identified in a yeast two-hybrid assay, two co-immunoprecipitated with FADD-DN in mammalian cells. The first is the death receptor DR5, which binds to the cytotoxic ligand TRAIL and the second is a protein with unknown function, PL31. We subsequently identified a mutation in FADD-DN that prevented interaction of these proteins with FADD-DN.

The scope for the third year of work was to determine which of these binding partners is involved in FADD-DN induced death. The approach was to use a yeast forward two-hybrid screen to make compensating mutations in FADD-DN binding partners that could restore interaction with a mutated FADD-DN. We tested the validity of this approach using Fas, which is a death receptor known to bind FADD-DN. Several mutations in FADD-DN that prevent interaction with Fas (R117A, D123R) were identified. Using FADD-DN (R117A) as bait, we performed a two-hybrid screen with randomly mutated Fas as prey and identified several mutant Fas molecules that could restore interaction with FADD-DN (R117A). These mutations in Fas could restore interaction with FADD in one of two ways. In the first scenario, the mutation in Fas was specific such that it compensated for the mutation in FADD-DN. In the second scenario, the mutation in Fas increases the affinity of the Fas - FADD-DN interaction and does not specifically compensate for the mutation in FADD-DN. To discriminate between these possibilities, we tested the mutant Fas molecules that restored interaction with FADD-DN (R117A) to determine whether this mutation also restored interaction with FADD-DN (D123R). If the compensating mutation were specific, interaction with D123R should not be restored. However, in a directed yeast two-hybrid, the mutations that restored binding to R117A also restored interaction with D123R. This indicated that our approach for finding

mutation in FADD interacting proteins that could restore binding to a mutant FADD-DN would not work.

To determine the signaling molecule involved in FADD-DN signaling, we focused our efforts on DR5 because experiments in our laboratory indicated that DR5 activated a death pathway in normal prostate cells similar to FADD-DN (Thorburn et al., 2005). This allowed us to use an alternative approach to identify FADD-DN mutations abrogated the FADD-DN signaling pathway. Using site directed mutagenesis, we made 18 YFP (Yellow Fluorescent Protein) tagged mutations in FADD-DN and tested each for its ability to interact with DR5 using a functional screen. Because YFP replaces the FADD death effector domain, these FADD molecules cannot recruit caspases to activated death receptors, and thus act as dominant negatives (Wajant et al., 1998). This allowed us to screen through these mutation and identify those that prevented interaction with DR5. The transfected HeLa cells were treated with an agonistic antibody that activates DR5 and cytotoxicity was measured after 24 hours. As shown in figure 1, seven of these mutations (D106A, R117A, D123R, R135E, R140A, R166E, and D175R) prevented interaction of FADD-DN with DR5.

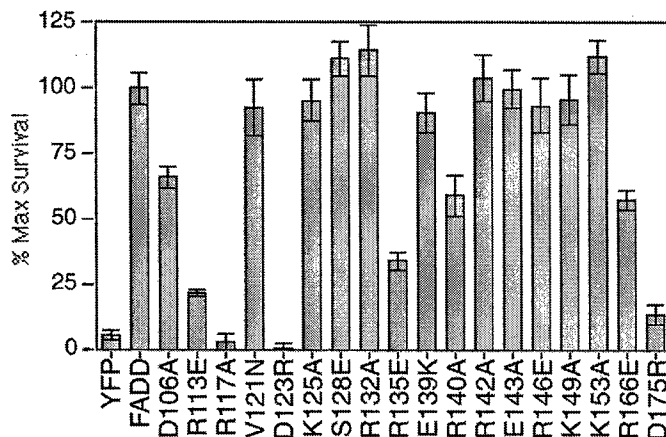


Figure 1: FADD-DN mutations prevent dominant negative activity *in-vivo*. HeLa cells expressing YFP, FADD-DN or each of our 18 YFP-tagged FADD-DN mutations were treated with a DR5 agonist that induces apoptosis. Cytotoxicity was determined after 24 hours. Several of the FADD-DN mutants decreased the ability of FADD-DN to inhibit DR5-induced apoptosis indicating that the mutations altered binding of FADD-DN to DR5.

To determine the efficacy of this approach, we tested each of the mutations that prevented dominant negative function by FADD-DN for interaction of FADD-DN with DR5 by immunoprecipitation. With the exception of D106A which had the smallest effect of all the FADD-DN mutations in the functional screen, all of the mutations that prevented FADD-DN function also prevented interaction of FADD with DR5 (fig 2). This indicated that our approach of identifying FADD-DN mutations that prevent interaction with DR5 was valid.

Given our previous experiments with DR5 (Thorburn et al., 2005), we reason that DR5 is likely the signaling receptor involved in FADD-DN induced death in normal prostate and mammary epithelial cells. Experiments using the identified FADD-DN mutations that prevent binding to DR5 will determine whether DR5 is the only protein involved. If all the mutations that prevent binding to DR5 also fail to kill normal mammary epithelial cells, then we will conclude that no other molecules are involved. If some of the FADD-DN mutants are still able to kill, then we will conclude that other signaling molecules are

involved. In this case we will determine whether these mutations prevent binding to other FADD-DN interacting partners such as PL31.

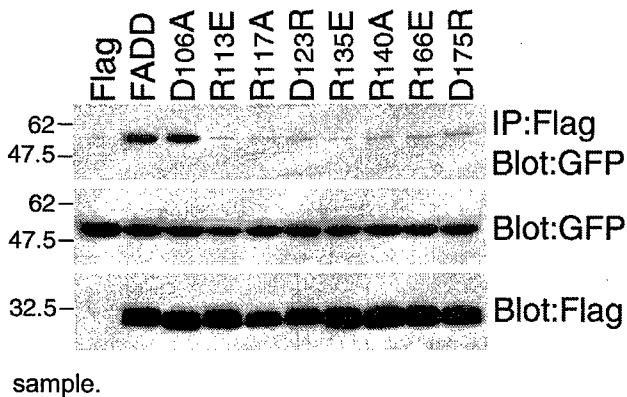


Figure 2: Mutations in FADD that prevent interaction with DR5. Flag-tagged FADD was co-transfected with GFP-tagged DR5 into HeLa cells and Flag complexes were precipitated. The ability of each FADD mutant to interact with DR5 was measured by immunoblotting for GFP. Whole cell lysate was immunoblotted with Flag and GFP antibodies to determine the amount of Flag-FADD and GFP-DR5 to show equal amounts of these proteins in each sample.

Finally, we have begun to move our findings toward a more clinical setting. We have obtained agonistic antibodies from Human Genome Sciences that activate DR5, which are currently in clinical trials to treat a variety of cancers. My former mentor is participating in the clinical trials in breast cancer patients at his new institution. Thus, we anticipate that our finding will help to understand fundamental differences in apoptotic signaling pathways between normal and cancerous breast cells.

Key Research Accomplishments

- We determined that our previously designed strategy of identifying the protein(s) involved in FADD-DN signaling was not viable.
- Using an alternate strategy, we generated 18 mutations in FADD-DN and identified seven that prevent interaction with DR5.
- Co-immunoprecipitation experiments were performed to identify FADD-DN mutations that prevented interaction with DR5.
- We constructed plasmids expressing these FADD-DN mutations, will be micro-injected into human breast epithelial cells to determine whether DR5 is the signaling molecule involved in FADD-DN induced death.

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Abstracts:

June 2004: The 20th Annual Meeting on Oncogenes. Somatic Mutations of the TRAIL Receptor DR5 Prevent FADD recruitment to the activated TRAIL Receptor Complex.

Conclusions

Because our initial strategy was not feasible for identifying compensating mutation in FADD-DN interaction proteins, we designed a second approach, which utilized a functional screen to identify mutations in FADD-DN that prevented interaction with DR5. Using this approach, we identified seven mutations that prevented FADD-DN function. These mutations will be tested for the ability to kill normal breast epithelial cells. In addition, we have also initiated experiments using agonistic DR5 antibodies that are in clinical trials to determine the efficacy of these antibodies to kill patient-derived tumor cells.

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